Functional Genomics of the Regulation of the Nitrate Assimilation Pathway in Chlamydomonas[1]

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The existence of mutants at specific steps in a pathway is a valuable tool of functional genomics in an organism. Heterologous integration occurring during transformation with a selectable marker in Chlamydomonas (*Chlamydomonas reinhardtii*) has been used to generate an ordered mutant library. A strain, having a chimeric construct (*pNia1*::*arylsulfatase* gene) as a sensor of the *Nia1* gene promoter activity, was transformed with a plasmid bearing the paramomycin resistance *AphVIII* gene to generate insertional mutants defective at regulatory steps of the nitrate assimilation pathway. Twenty-two thousand transformants were obtained and maintained in pools of 96 for further use. The mutant library was screened for the following phenotypes: insensitivity to the negative signal of ammonium, insensitivity to the positive signal of nitrate, overexpression in nitrate, and inability to use nitrate. Analyses of mutants showed that (1) the number or integrated copies of the gene marker is close to 1; (2) the probability of cloning the DNA region at the marker insertion site is high (76%); (3) insertions occur randomly; and (4) integrations at different positions and orientations of the same genomic region appeared in at least three cases. Some of the mutants analyzed were found to be affected at putative new genes related to regulatory functions, such as guanylate cyclase, protein kinase, peptidyl-prolyl isomerase, or DNA binding. The Chlamydomonas mutant library constructed would also be valuable to identify any other gene with a screenable phenotype.

The unicellular eukaryotic green alga Chlamydomonas (*Chlamydomonas reinhardtii*) has attracted the attention and effort of many scientists, leading to a set of effective molecular tools that, together with the classical physiological, genetic, and biochemical methods, allow one to study genes and unravel gene function in different biological processes (Lefebvre and Silflow, 1999; Grossman, 2000; Dent et al., 2001; Harris, 2001; León-Bañares et al., 2004). The potential of this organism as a model system has been boosted by the ongoing Joint Genome Institute (JGI) Chlamydomonas sequencing projects (http://genome.jgi-psf.org/chlre2/chlre2.home.html) and by the availability of over 200,000 Chlamydomonas expressed sequence tags (ESTs; www.kazusa.or.jp/en/plant/chlamy/EST and www.biology.duke.edu/chlamy_genome; Grossman et al., 2003; Shrager et al., 2003).

One of the biological processes for which Chlamydomonas is an excellent model system is mineral nutrition (Grossman, 2000; Galván and Fernández, 2001; Harris, 2001). Nitrogen assimilation is a highly regulated process that provides a pathway for incorporating the nitrogen macronutrient in an integrated manner with other carbon, sulfur, or phosphorus nutrients, light, and circadian rhythms (Grossman, 2000; Waltenberger et al., 2001; Crawford and Forde, 2002; Maathuis et al., 2003; Wang et al., 2003; Todd et al., 2004). Inorganic nitrogen also signals for modulation of different pathways and cell differentiation processes in plants (Crawford and Forde, 2002) and algae (Pozuelo et al., 2001). Specifically, the nitrate assimilation pathway has been dissected and most of the genes encoding its singular structural elements have been identified. Their gene expression is coordinately regulated with respect to the nitrogen source, the intracellular amounts of reduced nitrogen compounds, light, hormones, and carbon status (Hoff et al., 1994; Crawford, 1995; Crawford and Glass, 1998; Stitt et al., 2002). The information on regulatory genes for nitrate assimilation in plants and algae is scarce, and only the Chlamydomonas *Nit2* has been reported as a major positive regulatory gene for this pathway (Fernández and Matagne, 1986; Schnell and Lefebvre, 1993).

In an overall view of the regulation of nitrate assimilation in Chlamydomonas, expression of the genes in this pathway is subject to repression by ammonium and induction by nitrate (Quesada and Fernández, 1994; Fernández et al., 1998). The positive regulatory gene *Nit2* is itself repressed by ammonium, which may reflect an additional level of control (Schnell and Lefebvre, 1993). A locus for a second regulatory gene, *Nit9*, closely linked to *Nit2*, has also been reported (Rexach et al., 1999). By insertional mutagenesis, different negative regulatory loci (*Nrg1*-4, *Far1*) were reported to mediate ammonium repression with partial

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phenotypes for the nitrate transport and reduction steps (Prieto et al., 1996; Zhang and Lefebvre, 1997; Pérez-Alegre, 2003). The regulatory genes and mechanisms for the nitrate pathway in algae and plants appear to be different from those in fungi (Crawford and Arst, 1993; Marzluf, 1997). It has been proposed that several genes might mediate positive effects of nitrate and negative effects of ammonium so that their deficiency would lead to partial phenotypes. This proposal might explain the difficulties found in the genetic dissection of the regulation both in algae and plants (Galván and Fernández, 2001). In this context, functional genomics might provide a way to identify genes and their functions by using strategies of forward and reverse genetics.

Insertional mutagenesis in Chlamydomonas has proven to be useful in the cloning and analysis of genes involved in different biological functions. Several selectable markers are routinely used to obtain efficient transformations and screenings of particular phenotypes (forward genetics). The sequences affected at the insertion sites are then cloned by plasmid rescue (Tam and Lefebvre, 1993; Cenkci et al., 2003; Riekhof et al., 2003), inverse PCR (Pérez-Alegre, 2003; Yoshioka et al., 2004), or thermal asymmetric interlaced (TAIL)-PCR (Colombo et al., 2002; Pollock et al., 2003) approaches.

In this work, an ordered, insertionally tagged mutant collection from Chlamydomonas has been constructed mainly to identify by functional genomics genes involved in the regulatory circuits of nitrate assimilation. In this first approach, forward genetics screenings have been undertaken and sequences flanking insertion sites obtained, with the availability of the Chlamydomonas genomic sequence facilitating the identification of putative genes responsible for specific phenotypes.

RESULTS AND DISCUSSION

Construction of an Ordered Mutant Library from Chlamydomonas: Isolation of Regulatory Mutants

Insertional mutagenesis has been widely used in Chlamydomonas to obtain mutant strains and to identify the gene responsible for a mutant phenotype. In spite of its usefulness, this technique presents some difficulties due to the integration of multiple copies of the marker, loss of the tag, or problems in isolating the region adjacent to the insertion. In this work, we have tried to minimize these difficulties by setting up conditions for single integration of the tag and by improving the technique to identify insertion sites. For this purpose, we have constructed a Chlamydomonas mutant library from the 704 strain containing a chimeric construction of the *Nia1* gene promoter fused to the arylsulfatase (*Ars*) reporter gene (Loppes et al., 1999), which allows monitoring of the *Nia1* expression in response to its regulatory elements (Ohresser et al., 1997). This strain was mutagenized by random insertion of the pSI104 that contains the paramomycin resistance *AphVIII* gene (Sizova et al., 2001). Although a number of gene markers have been used in Chlamydomonas with different purposes (Grossman, 2000; León-Bañares et al., 2004), we have chosen *AphVIII* versus others to avoid problems related to the lack of tagging by the DNA insertion (Dent et al., 2001) or the appearance of spontaneous mutations in the selection media (as we found in bleomycin-containing media; D. González-Ballester, J. Rexach, A. Llamas, A. Galván, and E. Fernández, unpublished data).

The procedure followed to construct the collection of mutants, the setup for screenings, and the orderly groups of mutants are summarized in Figure 1. A library of about 22,000 mutants, ordered in 260 pools of 96 mutants, has been generated in a first step. Although this number does not seem to be high, it has to be considered that insertional mutagenesis causes deletions of DNA fragments from 5 to 57 kb (Kindle, 1998; Cenkci et al., 2003), which might make difficult the identification of the gene responsible for the phenotype, but in turn does have the advantage of a lower number of transformants to saturate the genome (Dent et al., 2001). This mutant library was screened for four different phenotypes: ammonium insensitive (AI); nitrate insensitive (NI); overexpressed in nitrate (ON); and no growth on nitrate (Nit^-; Fig. 2). These phenotypes would be some of the expected ones in mutants affected in the sensing of the strong repressor effect of ammonium or the requirement of nitrate for *Nia1* expression (Fernández and Cárdenas, 1989; Ohresser et al., 1997; Fernández et al., 1998; Llamas et al., 2002). Tests for growth on nitrate media together with the ARS reporter activity would be valuable to differentiate apparent regulatory effects that are simply due to the lack of nitrate reductase (NR) activity as previously reported (Loppes et al., 1999; Llamas et al., 2002).

AI Mutants

A total of 139 mutants were preselected for having ARS activity in the presence of ammonium; however, only 40 of them were confirmed as positive AI mutants after rescreening (Table I). To know the level of insensitivity to ammonium of these 40 mutants, ARS activity per cell number was more reliably measured in Tris-acetate phosphate (TAP)-ammonium-nitrate liquid medium after 3 to 4 d of growth and compared with those found in the parental strain 704 under the same conditions (Fig. 3). The AI mutants fit into three groups, depending on the level of activity detected: very insensitive to ammonium (from 30- to 60-fold higher ARS than the parental); insensitive to ammonium (around 10- to 20-fold higher); and moderately insensitive (2- to 5-fold higher). Four insertional mutants had previously been isolated in Chlamydomonas and found to be defective at four unlinked loci, named *Nrg* (Prieto et al., 1996; Pérez-Alegre, 2003). Three

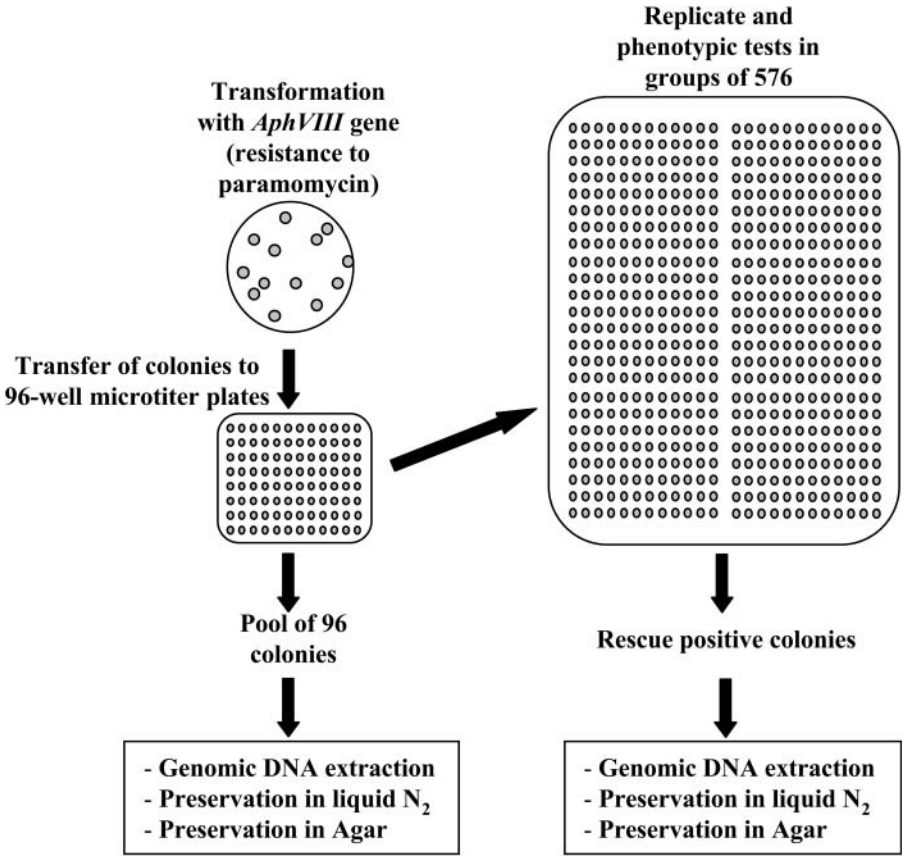

Figure 1. Scheme of steps followed for the construction of the Chlamydomonas mutant library.

of these mutants showed a partial phenotype of ammonium insensitivity that was additive in double mutants, whereas the fourth one had a strong phenotype of ammonium insensitivity. These facts indicate, in agreement with the results presented herein, a complexity in the network for sensing ammonium and its metabolic derivatives in the alga. Molecular identification of the genomic region affected by the marker insertion was performed for the 40 mutants and successfully obtained for 30 of them. The loci affected are shown in Table II and discussed below.

NI Mutants

These mutants were selected on the basis of their deficiency in ARS activity in TAP-nitrate-urea medium (Fig. 2). Nitrate is a positive signal, whereas urea provides a neutral signal for *Nia1* expression (Fernández et al., 1998) and allows growth of the possible Nit$^-$ mutants. From 204 preselected mutants, only 47 were confirmed by rescreening, 7 of which were Nit$^-$ mutants (Table I). All the NI Nit$^-$ mutants had a drastic loss of ARS activity, showing that they were drastically affected in nitrate assimilation and most probably in critical genes such as *Nit2* or new regulatory genes. In contrast, defects on genes related to NR enzyme structure or molybdenum cofactor (MoCo) biosynthesis should be intact, since defects on any of them would produce an overexpression of *Nia1* (see below). On the other hand, the NI Nit$^+$ mutants showed a weaker ARS activity than the wild type, which implies a decrease of the *Nia1* expression but a sufficient NR activity to grow in nitrate media. Molecular identification of genes affected was performed for only two NI Nit$^-$ mutants (Table II; see below). No molecular assays have been done so far over the NI Nit$^+$ mutants.

ON Mutants

To find genes whose defect produced an up-regulation of the *Nia1* expression and therefore could be implicated in the regulation of nitrate assimilation, we selected the ON mutants based on their high ARS activity in TAP-nitrate-urea medium compared to the wild type (Fig. 2). From 133 preselected mutants, only 58 were confirmed, 13 of which were Nit$^-$ (Table I). These ON Nit$^-$ mutants might be displaying the well-known phenomenon previously denominated autoregulation of *Nia1* (Fernández et al., 1998), which has been clearly shown to be an effect derived from the intracellular nitrate accumulation (Llamas et al., 2002). Thus, most of these mutants might not be affected at regulatory genes but in genes related to NR functionality. One of these mutants was found to be defective in a MoCo biosynthesis gene (Table II). No molecular assays have been carried out over any ON

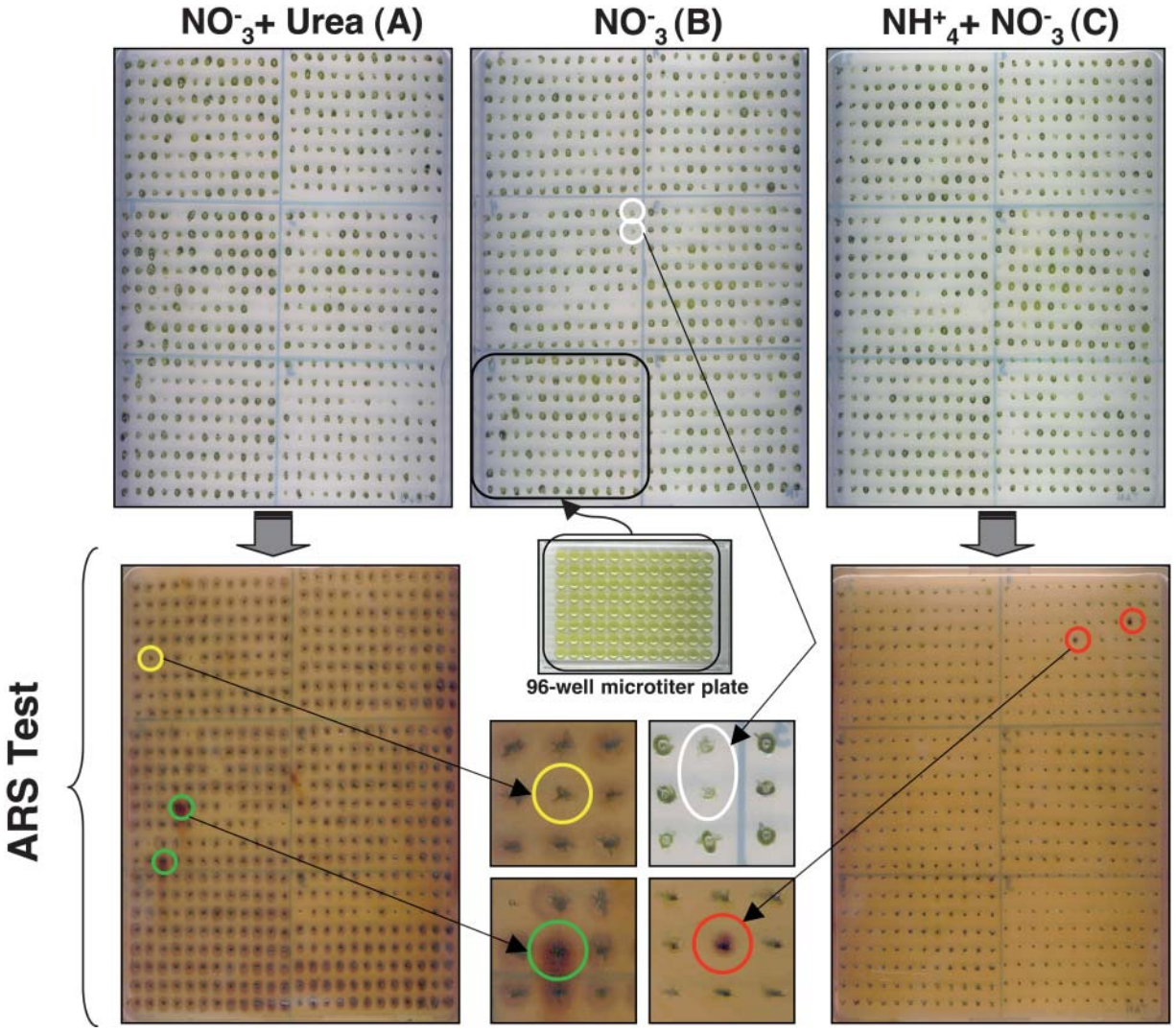

Figure 2. Screening procedure of mutants. From a 96-well microtiter plate, 3 replicates were done on different square plates containing the indicated nitrogen sources. ARS activity test was performed over plates A and C. NI and ON mutants were screened from plates A (yellow and green rings, respectively), whereas AI mutants were screened from plate C (red rings). Nitrate assimilation mutants (Nit^-) were observed over plate B (white rings). Examples of each phenotype are shown magnified.

Nit^+ mutants except for 106.20, which was also an AI mutant (see below).

Determination of the DNA Tag Copy Number

The transformation conditions were set up to reach an acceptable efficiency (1,000–5,000 transformants/microgram of DNA) together with single insertions of the DNA marker. Optimal conditions were found by using low concentrations of DNA and polyethylene glycol in the transformations. The copy number of 18 randomly isolated transformants was checked by Southern blot. As shown in Figure 4, a single copy of the *AphVIII* gene marker was present in each of them. In addition, most AI mutants were analyzed both by Southern blot and real-time PCR to determine the number of DNA tag copies. A single copy was found in most of them (Table III), with some exceptions. Mutant 47.72 showed about 4 copies by real-time PCR and a single strong hybridization signal band by Southern blot, suggesting that multiple copies of the marker gene had been integrated in tandem. Similarly, mutant 259.3 seemed to have two marker copies. Interestingly, in mutants 5.3, 7.81, 13.69, 106.20, and 213.94, copy number was almost undetectable by real-time PCR (100- to 1,000-fold less than the positive control), and they showed no hybridization signal (except 5.3). Difficulties in cloning the region adjacent to the insertion were also found in these strains, although molecular identification was successful in mutants 106.20 and 213.94. In addition, all these mutants had a normal growth in paramomycin medium during the first days after plating, but after 5 to 6 d they started to die. It is possible that the marker gene is undergoing silencing by epigenetic phenomena that modify the structure of the marker gene interfering

Table I. *Number and type of the selected mutants*

Activity of the *Ars* gene under control of the *Nia1* promoter is shown in the *pNia1*-Ars lines where +, −, and ++ represent presence, absence, and overexpression of activity in the appropriate screening media, respectively. Growth (+) or no growth (−) on nitrate media of the same strains is indicated in the Nit lines. AI, Ammonium insensitive mutants; NI, nitrate insensitive mutants; ON, overexpressed in nitrate mutants. –, No mutant of this type.

Phenotype for ARS Activity and Nit	Number of Isolated Mutants		
	AI	NI	ON
pNia1-Ars^{+}	40	–	–
pNia1-Ars^{-}	–	47	–
pNia1-Ars^{++}	–	–	58
Nit^{-}	–	7	13
Nit^{+}	40	40	45
Total		145	

with alignment of probes in Southern blots and of primers during real-time PCR determinations. These epigenetic phenomena in nuclear transformants have been reported and studied extensively in Chlamydomonas (Cerutti et al., 1997; Wu-Scharf et al., 2000).

Features of the Integration and Analysis of the Affected Loci

A modified TAIL-PCR technique (D. González-Ballester, A. de Montaigu, A. Galván, and E. Fernández, unpublished data) was used to identify both flanking regions of the plasmid insertions. We were mostly successful with primers from the *AphVIII* marker gene; however, we failed in amplifications of genomic regions at the other side of the marker close to the vector sequence. Two facts account for this failure. The first one is the presence of two other plasmid vectors in the parental strain 704 (bearing *Nia1*::*Ars* and *Arg-7* genes; Ohresser et al., 1997) that prevent the use of vector primers. The second is the presence of about 4 kb of vector sequence in the linearized pSI104 from the marker gene, which makes difficult amplifications with marker primers. So we know only the adjacent region situated close to the marker gene and we have no direct evidence of the sizes of the deletion or reorganization at these positions. In spite of this, we could verify that most of the integrations occurred close to the *Kpn*I site used to linearize the pSI104 plasmid. Only two of the analyzed mutants (21.88 and 106.20) had lost part of the 3′ untranslated region of the marker gene situated near the *Kpn*I site. From a total of 43 mutants, we could identify the insertion site sequence in 33 mutants, which represents about 75% success. Mutants 21.90 and 23.59 corresponded exactly to the same insertional mutation; likewise, it occurred with mutants 255.92 and 256.10. Thus, these mutants had been selected twice in the screenings because they came from the same transformation set, which might suggest a redundancy of about 5% in the mutant library.

Mutants at *Cnx2* and *Nit2* as Examples of the Mutant Library Usefulness

Three Nit^{-} mutants were used as examples to clone the region adjacent to the tag insertion and to validate the molecular identification and its correspondence with the observed phenotypes. These strains were chosen due to the well-known mutant phenotypes for nitrate assimilation (Fernández et al., 1998; Galván and Fernández, 2001). One of these Nit^{-} mutants, 7.91, showed an ON phenotype. According to our present knowledge, this mutant might bear defects at the NR structural gene *Nia1* or at MoCo biosynthesis genes leading to the lack of NR activity and to overexpression of nitrate-regulated genes (Quesada and Fernández, 1994; Llamas et al., 2002). In this mutant, sequence of

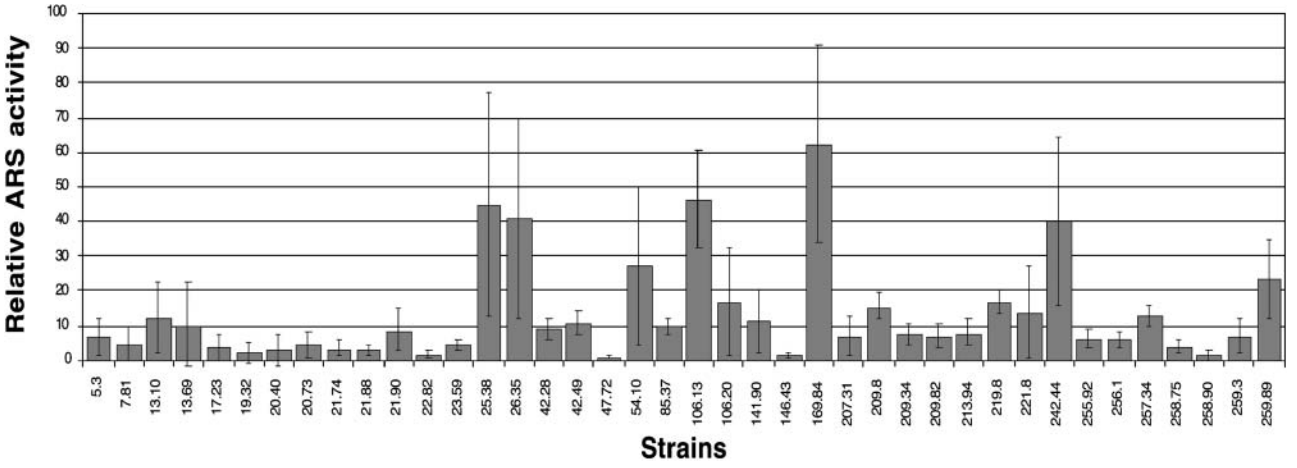

Figure 3. ARS activity in the AI mutants. Cells were grown in TAP-ammonium-nitrate media and measurements of the ARS activity were performed after 3 to 4 d of growth. ARS activity is determined as absorbance units per cell number of each culture and expressed as relative folds with respect to activity in the parental strain 704 under the same conditions. Data correspond to four independent determinations for each mutant.

Table II. *Molecular features of some insertional mutants*

Data relative to the positions, gene prediction, and homologies of the loci affected are described based on version 2 of the JGI Chlamydomonas genome server. Localizations of the insertions are described as follows: first in bold, numbers of scaffold and gene ID (when predicted by JGI) separated by two dots; second in parentheses, the scaffold position of the insertion with ← or → symbols indicating left or right direction of the vector, respectively; and, finally, the linkage group of the scaffold (when known) in roman numerals. GenBank NCBI accession numbers of the genomic sequence that flanked the DNA marker integrations are indicated in parenthesis below the names of mutants. Additional information about the real or putative locus affected is given just for those that are interrupted by the insertion. Unknown protein refers to putative predicted genes.

Mutant	Insertion Localization	Putative Genes Affected		Notes
		Description	Biological Function	
ON Phenotype				
7.91 (AY704181 and AY704182)	**2** (←25,100) and **801:C_8010002** (←5,710)	Cnx2	MoCo biosynthesis. Nitrate metabolism	Insertion located at 1.5 kb of *Cnx2*
NI Phenotype				
177.1 (AY704202)	**86** (109200→)/**III**	Nit2	Major nitrate regulatory gene	Insertion located around 2.5 kb of *Nit2* gene
257.67 (AY704210)	**2458:C_24580001** (274→)/**III**	Nit2	Major nitrate regulatory gene	In v.1 of JGI, insertion located around 15 kb of *Nit2* gene (scaffold 17)
AI Phenotype				
13.10 (AY704183)	**3** (243,560→)/**III**	EST presence		Same region as 22.82
17.23 (AY704188)	**34** (←389,800)	EST presence		
19.32 (AY704184)	**16** (←276,000)/**XII**	EST presence		
20.40 (AY704185)	**6:C_60145** (←1,340,280)/**X–XVIII**	Unknown protein (EST presence)		
20.73 (AY704186)	**65:C_650021** (291,300→)	Homology with human guanylate cyclase (NP000848; 18,2% identity)	Intracellular signaling cascade	
21.74 (AY704187)	**12:C_120097** (941,780→)	Homology with Chlamydomonas protein kinase (AAF97501; 20% identity)	Signal transduction mechanisms	
21.88 (AY704189)	**115:C_1150005** (←125,000)	Dhc1	Cytoskeleton	
21.90 (AY704190)	**73** (←214,370)/**IX**			
22.82 (AY704191)	**3** (←240,890)/**III**			Same region as 13.10
25.38 (AY704192)	**291** (6500→)			Entire sequence not available in JGI
42.49 (AY704193)	**6:C_60013** (←1,139,060)/**X–XVIII**	Unknown protein (EST presence)		
47.72 (AY704194)	**140:C_1400012** (201,650→) and **2079** (2870→)	Homology with human hsa:6421 SFPQ; splicing factor Pro/Gln rich (NP005057; 29% identity)	mRNA maturing	Two scaffolds with very similar sequences
54.10 (AY704195)	**48** (585,760→)/**XI**	EST presence		Same region as 242.44
85.37 (AY704196)	**49:C_490059** (79,700→)/**XII**	Protein with DNA-binding motif (BRIGHT/ARID domain)	DNA binding	Same locus as 255.92 mutant
106.13 (AY704197)	**112:C_1120009** (196,360→)	Homology with EBNA-1 nuclear protein of human herpesvirus 4 (P03211; 18% identity)	DNA binding	
106.20 (AY704198)	**48:C_480037** (560,410→)/**XI**	Homology with *Fkb2* of Arabidopsis; PPIase, FKBP-type, rotamase (Q38935; 52% identity)	Protein folding: posttranslational modification, protein turnover, chaperones	Region very close to those of 54.10 and 242.44 mutants. This mutant has also an ON phenotype

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Mutant	Insertion Localization	Putative Genes Affected		Notes
		Description	Biological Function	
146.43 (AY704200)	**92:C_920053** (←384,230)	Ars		Endogenous *Ars* gene promoter is affected
169.84 (AY704201)	**11** (←580,870)/**III**			
207.31 (AY704203)	**80:C_800034** (←418,580 or ←425,460)/**X**	Unknown protein (EST presence)		Repetitive sequence in the same scaffold
209.34 (AY704204)	Unknown			No sequence available in JGI
209.82 (AY704205)	**21:C_210095** and **C_210005** (←1,044,850)/**IX–XIV**	Unknown protein		
213.94 (AY704206)	**2**: **C_20351** (←743,360)/**II**	Unknown protein (EST presence)		
219.8 (AY704207)	**6:C_60085** (699,600→)/**X–XVIII**	Homology with putative plant mitochondrial carrier proteins (EST presence)	Mitochondrial substrate carrier	
242.44 (AY704208)	**48:C_480041** (589,690→)/**XI**	Unknown protein		Same region as 54.10 mutant
255.92 (AY704209)	**49:C_490059** (←82,420)/**XII**	Protein with DNA-binding motif (BRIGHT/ARID domain)	DNA binding	Same locus as 85.37 mutant
258.90 (AY704211)	**156:C_1560006** (←15,250)	Homology with a PPIase, cyclophilin type of Arabidopsis (Q42406; 46% identity)	Protein folding: posttranslational modification, protein turnover, chaperones	
259.89 (AY704212)	**35:C_350113** (623,140→)/**I**	Homology with ubiquitin-protein ligase genes	Protein modification, ubiquitin cycle	

the cloned region adjacent to the insertion showed that the marker gene had been integrated in a location very close (at about 1.5 kb) to the 5′ end of a putative Chlamydomonas *Cnx2* gene that would have caused its deletion (Table II). By using specific primers, we found that the *Cnx2* coding region was in fact deleted in the mutant (data not shown). *Cnx2* is involved in the molybdopterin biosynthesis of MoCo, which is necessary for NR activity (Mendel and Schwarz, 1999). Thus

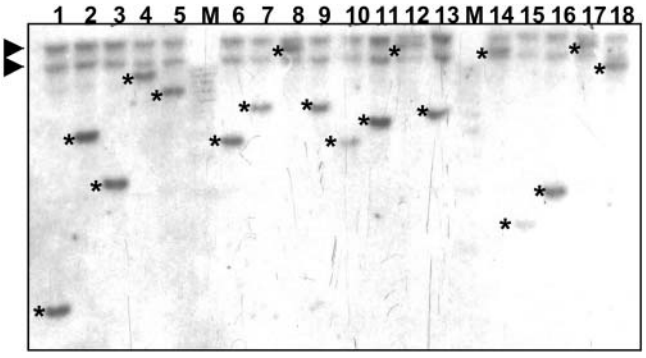

Figure 4. Determination by Southern blot of marker gene copy number. Genomic DNAs from 18 randomly isolated paramomycin-resistant mutants were digested with *Pst*I. Asterisks indicate the specific hybridization bands corresponding to *AphVIII* insertions. Triangles indicate two unspecific hybridization bands present in all the mutants. M, Lane of DNA size marker.

mutant 7.91 shows the expected correlation between the phenotype ON Nit^- and the gene function.

The other two Nit^- mutants (177.1 and 257.67) analyzed were NI. According to our present knowledge, these mutants might result from defects at the major and positive regulatory gene *Nit2* (Fernández and Matagne, 1986; Schnell and Lefebvre, 1993) or at other new genes. Isolation of the regions adjacent to the marker showed that insertions had taken place at 2.5 (mutant 177.1) and 15 kb (mutant 257.67) from the 5′ end of the *Nit2* gene. In addition, strain 177.1 and a *Nit2* mutant did not show positive complementation in diploids, suggesting that the *Nit2* gene is defective in this mutant. In fact, the wild-type phenotype was recovered when it was transformed with the pMN68 plasmid bearing the *Nit2* gene (Schnell and Lefebvre, 1993).

The molecular characteristics of these three Nit^- mutants provide strong evidence for connecting the phenotypes selected in the mutant library to the corresponding defective gene at the insertion site of the tag.

Molecular Characterization of AI Mutants

The localization of the insertion sites of the *AphVIII* marker in the 27 AI mutants analyzed is given in Table II. For several mutants, the integration has occurred within a gene or a putative gene that is also indicated.

Table III. *Determinations of AphVIII gene copy number*

The marker gene copy number was deduced from hybridization bands in Southern blot and from real-time PCR determinations as detailed in ''Materials and Methods.'' Multicopy control is indicated as 259.89 (2×). ND, Not detected; NA, not analyzed. Text in bold indicates mean values ± SD.

Mutant	Copy Number		Mutant	Copy Number	
	Southern	Real-Time		Southern	Real-Time
704	0	0	54.10	1	**1.00** ± 0.56
259.89	1	1	85.37	1	**1.52** ± 0.01
259.89 (2×)	-	**2.13** ± 0.28	106.13	1	**1.29** ± 0.04
5.3	1	**0.00063** ± 0.0003	106.20	ND	**0.0026** ± 0.0007
7.81	ND	**0.00075** ± 0.0005	141.90	1	**0.97** ± 0.44
13.10	1	**0.85** ± 0.08	146.43	1	**1.27** ± 0.42
13.69	ND	**0.00079** ± 0.0003	169.84	1	**1.03** ± 0.35
17.23	1	**0.85** ± 0.24	207.31	1	**0.97** ± 0.52
19.32	NA	**0.74** ± 0.004	209.8	1	**1.65** ± 0.31
20.40	1	**0.68** ± 0.003	209.34	1	**1.26** ± 0.46
20.73	1	**0.64** ± 0.019	209.82	1	**1.21** ± 0.42
21.74	NA	**1.56** ± 0.58	213.94	ND	**0.00095** ± 0.0002
21.88	1	**1.19** ± 0.32	219.8	1	**1.36** ± 1.19
21.90	1	**1.3** ± 0.50	221.8	1	**1.24** ± 0.55
22.82	1	**0.90** ± 0.39	242.44	1	**1.14** ± 0.74
23.59	1	**0.96** ± 0.30	255.92	1	**1.53** ± 0.17
25.38	1	**0.97** ± 0.28	256.10	1	**1.34** ± 0.36
26.35	1	**0.99** ± 0.65	257.34	1	**1.38** ± 0.28
42.28	1	**0.66** ± 0.01	258.75	NA	**1.37** ± 0.47
42.49	1	**0.71** ± 0.42	258.90	1	**1.42** ± 0.27
47.72	1	**4.1** ± 1.37	259.3	1	**2.00** ± 0.21

Nevertheless, for other mutants, no apparent genes seem to exist exactly at the marker integration point but close to it. However, these putative genes affected are not indicated because we have no direct evidence of the size of the deleted or reorganized genomic area.

In contrast to known nitrate assimilation mutants, no clues are available on the nature of genes expected to be responsible for the AI phenotype in algae or plants. Thus, at present, we have no direct evidence that interrupted genes in most AI mutants correlate with the observed phenotype, so further analyses are necessary to determine the gene responsible for the phenotype. Nevertheless, it is remarkable that some of the interrupted genes found for the AI phenotype are related to regulatory functions (Table II): transcriptional modulation (DNA-binding proteins); posttranscriptional regulation (mRNA splicing factor); posttranslational regulation (kinases and peptidyl-prolyl isomerases [PPIases]); protein degradation (ubiquitin ligase); and intracellular molecular signal biosynthesis (guanylate cyclase). The identification of two homologous genes for PPIase (or rotamase activity) from two different families (FKBP and cyclophilin types) is particularly interesting. These proteins are involved in protein folding and may also play important roles in mRNA processing, protein degradation, and signal transduction pathways implicated in both development and stress response (Fischer and Schmid, 1990; Romano et al., 2004). The loss of these general functions could have critical effects on gene regulation.

The existence of mutants that had been affected at the same loci by different and independent DNA integration events is noteworthy, giving strong evidence on which gene is responsible for the observed phenotype. In addition, these results confirm the validity of the screening strategy, the mutants selected, and the relationship between the mutations and the insertions. Three different examples of this situation are shown in Figure 5. The first case corresponds to AI mutants 54.10 and 242.44, and probably 106.20 (Fig. 5A). The first two have independent integrations very closely situated in the genome (about 3.9 kb apart), while 106.20 has a more distant insertion at about 25 kb. These three mutants have the integration in the same orientation, which makes difficult the delimitation of a possible genomic area responsible for the phenotype. Nevertheless, the importance of these DNA insertions in the AI phenotype seems evident. A second example refers to AI mutants 13.10 and 22.82, which show inverted orientations of the marker gene insertions that allow delimiting an area of about 2.5 kb in scaffold 3 with a high probability of being responsible for the phenotype (Fig. 5B). In this position, two genes are predicted (IDs C_30214 and C_30071), one of which is supported by the presence of ESTs. They would codify unknown proteins bearing one to two transmembrane domains. Finally, the case of 85.37 and 255.92 mutants is very interesting, where the gene responsible for the phenotype is clearly identified (Fig. 5C). In these mutants, the integrations occurred exactly in the same locus (gene ID: C_490059) and with different orientations, which would strongly suggest that a single gene is responsible for the observed phenotype. This candidate gene would codify

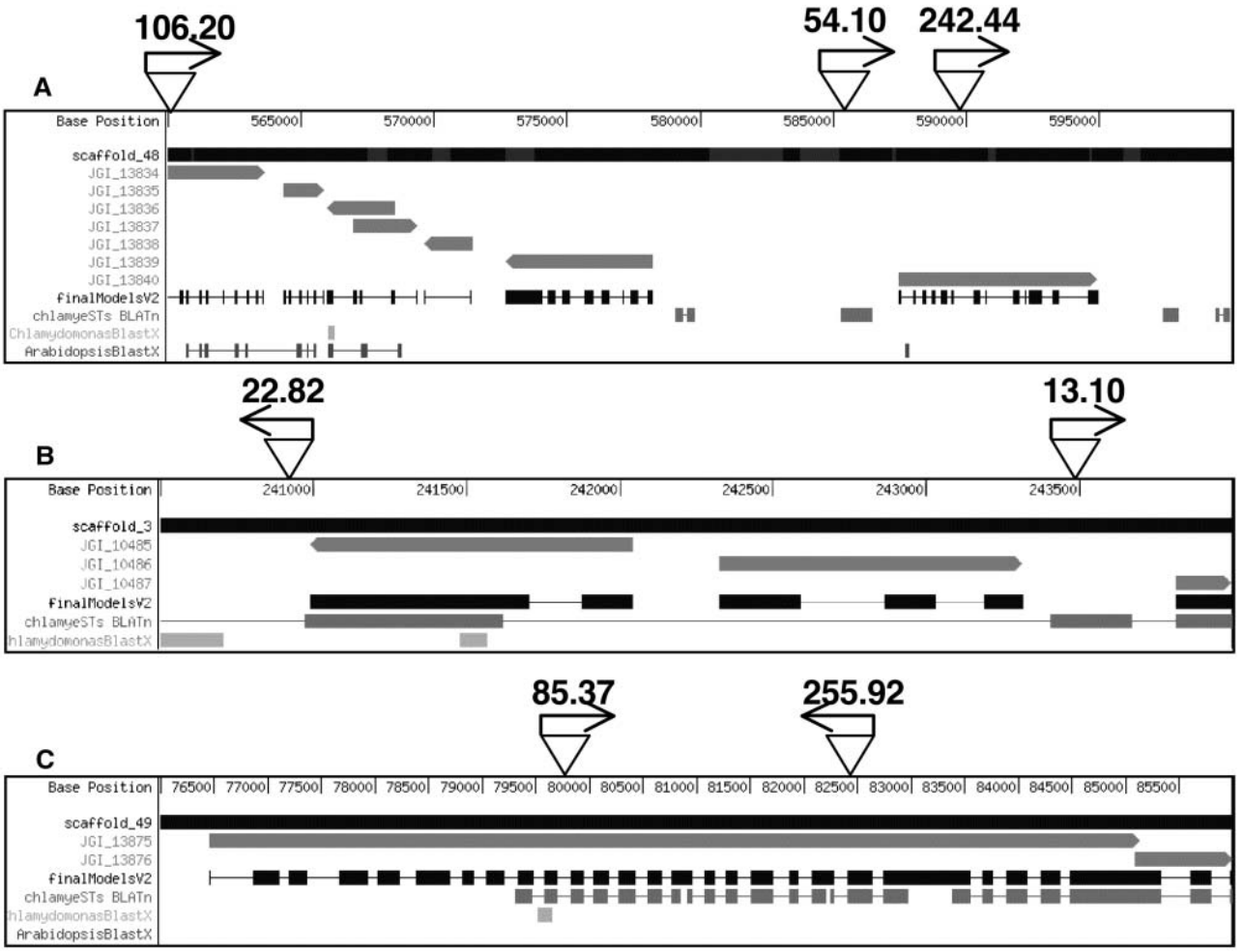

Figure 5. Schematic representation of the localization of three different linked mutations. Triangles represent the point of insertion and arrows the direction of the plasmid indicating the beginning of the putative deletions for each mutant. Map images of the genomic sequences are from the JGI Chlamydomonas genome server for the corresponding scaffold numbers: 48 (A), 3 (B), and 49 (C).

an unknown protein, supported by ESTs, containing a clear BRIGHT/ARID domain involved in DNA binding and transcription modulation (Wilsker et al., 2002). In addition, in two of the groups described above, ARS activity of the mutants is similar (Fig. 3), showing that mutations at the same locus have a similar consequent phenotype.

One false-positive AI mutant, strain 146.43, has been obtained. In this strain, insertion occurred at the 5′ end of the endogenous *Ars* gene so that its expression could have been placed under the control of the *rbcS-hsp70* chimeric promoter of the marker gene. For some mutants (17.23, 19.32, 20.40, 21.90, 25.38, 42.49, 169.84, 209.34, 209.82, 213.94, and 219.8; Table II), the information on possible genes affected in the region of the insertion is very scarce (presence of ESTs, putative encoded proteins) or simply nonexistent because some parts of the Chlamydomonas genome have not yet been sequenced or assembled (Shrager et al., 2003).

According to the data of the mutants analyzed in this work (Table II), insertions of the marker gene have occurred at different scaffold and linkage groups (when known), indicating a random and nondirected mechanism for the insertional mutagenesis in Chlamydomonas, which validates the potential of this mutant library for covering the genome and identifying specific genes.

The Chlamydomonas genome project is an invaluable tool for developing an insertional mutant library and analyzing putative regulatory genes for nitrate assimilation, which we have approached herein by forward genetics. The development of reverse genetics tools on this mutant library will be useful for defining functions of specific tagged genes. This is the main reason for having obtained this library in an ordered way. Although this library has been designed for identifying genes related to the regulation of nitrate assimilation, it will also be useful to identify nonessential genes related to any cell or metabolic process.

For these purposes, our mutant library will be available to the Chlamydomonas community.

CONCLUSION

An ordered Chlamydomonas insertional mutant library has been constructed and allowed to identify potential genes related to the regulation of nitrate assimilation by a forward genetics strategy. The mutants in the library contain mostly single-marker gene tags, distributed randomly along the genome, that are amenable for identifying the insertion site sequence with a fairly high probability. The mutant library might be a very useful tool for identifying all regulatory genes involved in positive and negative regulation of the nitrate assimilation pathway after increasing the number of mutants so that the genome is saturated by deletions/insertions. Any nonessential gene with a screenable phenotype could be isolated. The ordered pools of mutants will also allow developing reverse genetics strategies on the obtained library. Finally, putative genes identified from isolated AI mutants strongly suggest that a complex network of signaling proteins mediates the effects of ammonium and its derivatives on the nitrate assimilation pathway.

MATERIALS AND METHODS

Strains and Culture Conditions

In this work, the Chlamydomonas (*Chlamydomonas reinhardtii*) strain 704 (*cw15 arg7*$^{+}$ *Nia1:Ars mt*$^{+}$) (Loppes et al., 1999) was used as the parental type for insertional mutagenesis. Mutant 203d (*Nit2*$^{-}$ *mt*$^{-}$) was used in complementation analysis carried out as described by Fernández and Matagne (1986). Cells were cultured under continuous light at 23°C in liquid and solid TAP media (Harris, 1989) containing different nitrogen sources: TAP-ammonium-nitrate (7.5 mM ammonium chloride and 4 mM potassium nitrate); TAP-nitrate (4 mM potassium nitrate), TAP-nitrate-urea (4 mM potassium nitrate and 2 mM urea); and TAP-N (nitrogen-free). Where indicated, paramomycin was used at 25 μg/mL.

Generation of Insertional Mutants from Chlamydomonas

The cell wall-less strain 704 was efficiently transformed by the glass bead method (Kindle, 1990) with some modifications: polyethylene glycol 8000 was used at a final concentration of 2.5% (w/v); about 2×10^8 cells were shaken for only 8 s, and DNA concentration was adjusted to 100 ng/transformation reaction to avoid multiple and tandem copy insertions. The selectable marker employed was the *AphVIII* gene, which conferred resistance to paramomycin, under control of the *rbcS-hsp70* chimeric promoter (Sizova et al., 2001). This construct was present in the pSI104 plasmid derived from pSI103 (Sizova et al., 2001) after digestion with *Not*I and religation. The transformations were performed with pSI104 linearized by *Kpn*I digestion. After transformations, cells were incubated in TAP-N for 6 to 12 h under continuous light to induce APHVIII protein expression. Finally, transformants were selected in TAP-ammonium-nitrate medium supplemented with paramomycin. Colonies appearing on the selection plates at about 7 to 10 d in the light were transferred to 96-well microtiter plates with TAP-ammonium-nitrate liquid media and processed as described below.

Generation and Screening of an Ordered Mutant Library

A scheme for the mutant library construction is shown in Figure 1. From 96-well microtiter plates, after 2 to 3 d of growth, we made 3 replicates in 245- × 245-mm-square plates (Corning, NY), with different agar media using a 96-pin well replicator (Boekel Scientific, Feasterville, PA). In each square plate we set a total of 576 colonies from 6 different 96-well microtiter plates. Those agar media contained TAP-ammonium-nitrate, TAP-nitrate, and TAP-nitrate-urea (Fig. 2). After growth, ARS activity was measured directly on the square plates with TAP-ammonium-nitrate and TAP-nitrate-urea (Ohresser et al., 1997). Those mutants selected as putative regulatory mutants were rescreened in new agar plates to confirm the ARS test. An extra replica onto a 120-mm petri dish with TAP-ammonium-nitrate plus paramomycin, checking their similar growth, was made for each 96-well microtiter plate to recover the desired mutants and to make a pool of 96 individual mutants for further screenings (Fig. 1). These 96-mutant pools were made by adding 20 mL of TAP-ammonium-nitrate medium on the petri dish, gently shaking, and using the liquid medium to inoculate individually TAP-ammonium-nitrate medium. After 1 to 2 d of growth, these pools were both frozen in liquid nitrogen and preserved on agar medium, and an aliquot was taken to isolate genomic DNA. The mutants were named on the basis of 2 numbers separated by a point; the first one corresponds to the 96-mutant pool number and the second to the position in the master plate used to prepare this pool.

ARS Activity

ARS activity was determined directly on the agar plates after removing cells from the agar surface with a razor blade and following the procedure previously reported (Ohresser et al., 1997). ARS activity was also measured, in some mutants, in liquid media (TAP-ammonium-nitrate) as previously described (Ohresser et al., 1997; Llamas et al., 2002). ARS activity is expressed as absorbance units per cell number relative to the activity value obtained in the parental strain. Thus, ARS activity in the presence of ammonium in the parental strain (Llamas et al., 2002) is assigned a relative value of 1. The data presented in this work corresponded to mean values and SDs from at least three independent experiments.

Cryopreservation of Chlamydomonas in Liquid Nitrogen

Cells were frozen by the procedures previously reported by Crutchfield et al. (1999) and Sayre (http://www.biology.duke.edu/chlamy/methods/freezing.html) at the Chlamydomonas Genetics Center with some modifications. An average of 3.3×10^6 cells/mL were mixed in a 2-mL cryovial with methanol (3% final concentration in TAP-ammonium-nitrate medium) in a total volume of 1.8 mL. Cryovials were incubated in the dark at 4°C for 4 h and then placed at −80°C for 4 h in a freezing container (cryo 1°C freezing container; Nalgene, Rochester, NY, catalog no. 5100). The cryovials were transferred directly to liquid nitrogen. To thaw cells, cryovials were warmed at 35°C for 3 to 4 min and an aliquot (500 μL) was used to inoculate fresh TAP-ammonium-nitrate media. These cultures were kept for 12 h in the dark and then 1 to 2 d in dim light prior to growth under usual light conditions. To determine the rate of cell survival, the number of colonies formed on TAP-ammonium-nitrate plates from dilutions of thawed cultures was compared to the cell number counted in a microcell counter (Sysmec F-500) from the same cultures before the freezing treatment. Cell survival was around 3% to 6%, and no apparent decrease was observed after 1 year of freezing.

Real-Time PCR

Real-time PCR was performed on the LightCycler Instrument (iCycler iQ real-time PCR detection system; Bio-Rad, Hercules, CA) using SYBR Green I (10,000× concentrated in dimethyl sulfoxide (DMSO) according to the manufacturer; Molecular Probes, Leiden, The Netherlands) as a fluorescent dye. Each individual reaction was made in a 25-μL final volume with the following components: 0.2 pmol of each primer; 0.2 mM dNTPs; 0.5 units *Taq* DNA polymerase from Biotools (B&M Labs, Madrid); 2 mM $MgCl_2$; 1 to 5 ng of DNA; 1.25 μL of SYBR Green (diluted 10^{-4} in DMSO); 2.5 μL of the specific buffer, and MilliQ water up to 25 μL. The LightCycler-run protocol was 95°C, 5 min; 40× (95°C, 30 s; 63°C, 30 s; 72°C, 15 s; and fluorescence measurement at 84°C, 10 s, to avoid dimers and background signals). The specificity of the PCR amplification was checked by a melting curve program (60°C–100°C, with a heating rate of 0.5°C/s and a continuous fluorescence measurement) and analysis on 4% agarose gel electrophoresis.

To determine the number of integrations, we compared amplifications of the paramomycin resistance gene *AphVIII* with the endogenous *Nia1* gene (NR) as a single-copy gene control. Specific primers for the *AphVIII* gene were

5′-GAGGATCTGGACGAGGAGCGGAA-3′ (upper) and 5′-CCCTCAGAA-GAACTCGTCCAACAGC-3′ (lower); and for the *Nia1* gene 5′-GCGCTGCC-CTCCGTCACCTTCC-3′ (upper) and 5′-CAGCCGCACGCCCGTCCAGTAG-3′ (lower).

Efficiency of both pairs of primers was determined from its optimal annealing temperature by calculating the slope of a standard dilution curve, according to the method of Rasmussen (2001): 104% for *AphVIII* primers and 99.5% for *Nia1* primers. Due to the very close amplification efficiencies of both primer pairs, the number of integrations was calculated according to the Δ-ΔCt method (Ingham et al., 2001). We used the same amounts of DNA for *AphVIII* and *Nia1* gene amplifications for each mutant. One mutant (named 259.89) was used as a reference control whose copy number was found to be 1 by Southern blot. As a negative control, the parental wild-type strain 704 was used. As a multicopy positive control for *AphVIII* gene amplification, we used a double DNA amount from mutant 259.89.

The amplification rate of each transcript (Ct) was calculated by the PCR baseline subtracted method performed with LightCycler software (iCycler iQ, Optical System Software, v.3) at a constant fluorescence level. Cts were determined over three repeats within the LightCycler and with three different runs.

Identification of DNA Regions Flanking the Insertion

Genomic DNA adjacent to one border of the plasmid pSI104 insertion was isolated by a procedure modified from TAIL-PCR using specific primers from the marker gene sequence together with degenerated primers (Liu et al., 1995; D. González-Ballester, A. de Montaigu, A. Galván, and E. Fernández, unpublished data). *AphVIII* specific primers used were 5′-AGCTGGCCCAC-GAGGAGGAC-3′ and 5′-TACCGGCTGTTGGACGAGTTCTTCTG-3′. DNA sequences were directly obtained from isolated PCR-amplified fragments (SCAI Sequencing Facility, University of Córdoba) and analyzed in the Chlamydomonas JGI (http://genome.jgi-psf.org/chlre2/chlre2.home.html) and National Center for Biotechnology Information (NCBI) databases.

PCR Amplification of *Cnx2*

PCR amplifications of a *Cnx2* gene region were performed using the specific primers 5′-GTGAGGGCATGGCGGCGAGGACTG-3′ (upper) and 5′-GCAAGGCGTAAAGGAGGGGAGAAGGA-3′ (lower) under the following conditions: 95°C, 5 min; 35× (95°C, 30 s; 60°C, 30 s; 72°C, 1 min) in the presence of 2% DMSO.

Southern-Blot Analysis

Isolation of genomic DNA, electrophoretic fractionation of DNA, Southern transfers, hybridizations, and washes were performed as previously described (Sambrook et al., 1989; Quesada et al., 1993). In Southern blots, the specific probe for *AphVIII* was labeled either by random priming with α-[^{32}P]dCTP (a 750-bp *Ava*I-*Bam*HI fragment) or by PCR with dUTP-digoxigenin according to the DIG System user's guide (Boehringer Mannheim, Basel) and using the same primers as in the real-time PCR experiments (see above).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers listed in Table II.

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